Appendix A

OP-1 expression in transfected BSC cells may be induced by shifting the temperature down to 33°C in media containing 10% FCS, and harvesting the conditioned media after 96 hrs of incubation. Comparable amounts of OP-1 mRNA and protein are obtained, as compared with CHO cells (e.g., 100-150 ng OP-1/ml conditioned media from BSC clones transfected with pH717, see Oppermann et al.).

(3) CHO Cells

CHO cells (chinese hamster ovary cells) may be used for long term OP-1 production and are the currently preferred cell line for mammalian cell expression of OP-1. CHO cell lines are well characterized for the small and large scale production of foreign genes and are available commercially. See Oppermann et al., U. S. Patent No. 5,354,557, incorporated herein by reference, for a detailed description of: establishing a stable transfected cell line with high hOP-1 expression levels, subcloning transfected cells to obtain high expression subclones, characterizing subclone DNA insert copy numbers, and screening subclones for OP-1 mRNA and protein expression levels. Oppermann et al. also provides a detailed description of a rapid purification method for obtaining recombinantly produced

OP-1 of about 90% purity, and further data demonstrating the physical characteristics (molecular weight and glycosylation profiles) and osteogenic activities of a variety of recombinant forms of OP-1 expressed in the cell lines described above.

Accordingly, it is anticipated that active mature OP-1 sequences, including full-length, truncated and mutationally-altered active forms of the protein, can be expressed from other different prokaryotic and eukaryotic cell expression systems using procedures essentially as described herein. The proteins produced may have varying N-termini, and those expressed from eukaryotic cells may have varying glycosylation patterns. Finally, it will also be appreciated that these variations in the recombinant osteogenic protein produced will be characteristic of

the host cell expression system used rather than of the protein itself.

Example 3: **Synergistic Effect of Exogenous IGFI on the OP-1-induced Differentiation of Fetal Rat Calvarial (FRC) Cells**

Primary osteoblast cell cultures were prepared from fetal rat calvaria using published procedures (M.A. Aronow et al., J. Cell Physiol., 143, pp. 213-221 (1990); T.K. McCarthy et al., J. Bone Miner. Res., 3, pp. 401-8 (1988)). Briefly, cells were harvested by sequential collagenase digestions of the calvarium and cells from digestions III to V were pooled. Fetal rat calvaria (FRC) cells were plated in complete medium (MEM, alpha; GIBCO/BRL, Grand Island, NY) containing 10% fetal bovine serum, vitamin C (100μg/ml), and antibiotics (100 U/ml penicillin, and 100 mg/ml streptomycin). Cultures were incubated at 37°C with 95% air/5% CO_2 for several days to reach confluence. Cells were then subcultured for experimentations.

FRC cells were subcultured in 48-well plates (COSTAR, Cambridge, MA) in complete MEM medium with 10% fetal bovine serum until confluent in about 4 days. Confluent cells were rinsed with Hank's balanced salt solution (HBSS) and treated with serum-free α-MEM medium (with 0.1% BSA, 100 U/ml penicillin, and 100 mg/ml streptomycin) containing the appropriate solvent vehicle (50% acetonitrile/0.1% trifluoroacetic acid for OP-1 treatment or 0.1N acetic acid for IGFI treatment) or recombinant human OP-1, or IGFI at the concentrations indicated. Solvent vehicle concentration in the culture medium never exceeded 0.1%. At the end of treatment, cells were lysed and total cellular alkaline phosphatase activity was measured (typically after 48 hours of treatment).

Confluent FRC cells ($6\text{-}8\times10^6$ cells/T-150 flask) were rinsed once with HBSS to remove the complete medium and then incubated in serum-free α-MEM medium (with 0.1% BSA, 100 U/ml penicillin,

and 100 mg/ml streptomycin) in the presence or absence of OP-1 for varying intervals. OP-1 was dissolved in 50% acetonitrile and 0.1% trifluoroacetic acid (TFA). At the end of treatments, cells in the T-150 flask were rinsed with ice-cold PBS solution to remove serum-free medium and used for subsequent RNA isolation.

Alkaline phosphatase Activity Assay

Total cellular alkaline phosphatase activity was determined using a commercial assay kit (Sigma, St. Louis, MO). Cell lysates were prepared by aspirating the medium from the 48-well plate, rinsing the cells with ice-cold PBS, and lysing the cells with 0.05% Triton X-100 and sonication for 60 sec. Alkaline phosphatase activity in the lysates was measured in 2-amino-2-methyl-1-propanol buffer (pH 10.3) with p-nitrophenyl phosphate as substrate at 37°C. Reactions were performed in 96-well plates for 1-2 h. Following color development, reactions were terminated with 0.5N NaOH. Absorbance of the reaction was measured at 405 nm using a Hewlett Packard Genenchem automatic plate reader. Total protein level in the lysates was measured according to Bradford (M. Bradford, Anal. Biochem., 72, pp.248-54 (1976)) using bovine serum albumin as a standard. Alkaline phosphatase activity was expressed as nmol p-nitrophenol liberated per microgram of total cellular protein.

RNA isolation

Total RNA was isolated with cold Utraspec (Biotecx Lab., Houston, TX) following the manufacturer's recommendation. RNA was recovered by precipitation and dissolved in DEPC-H_2O. The amount of RNA recovered was estimated by A_{260} reading. The integrity of the RNA